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Human Endothelial Cells Selectively Express Large Amounts of Pancreatic-Type Ribonuclease (RNase 1)

Julien B.P. Landré,[1] Peter W. Hewett,[2] Jean-Marc Olivot,[3] Peter Friedl,[4] Yon Ko,[5] Agapios Sachinidis,[5] and Michel Moenner[6]*

[1]INSERM EPI-0113, Université Bordeaux-I, Avenue des Facultés, Talence, France
[2]Reproductive and Vascular Biology, The Medical School, University of Birmingham, Birmingham, United Kingdom
[3]INSERM U428, Université René Descartes-Paris V, Paris, France
[4]Institut für Biochemie, Technische Hochschule Darmstadt, Darmstadt, Germany
[5]Center of Physiology and Pathophysiology, Cologne, Germany
[6]INSERM U427, Université René Descartes-Paris V, Paris, France

Abstract Pyrimidine-specific ribonucleases are a superfamily of structurally related enzymes with distinct catalytic and biological properties. We used a combination of enzymatic and non-enzymatic assays to investigate the release of such enzymes by isolated cells in serum-free and serum-containing media. We found that human endothelial cells typically expressed large amounts of a pancreatic-type RNase that is related to, if not identical to, human pancreatic RNase. This enzyme exhibits pyrimidine-specific catalytic activity, with a marked preference for poly(C) substrate over poly(U) substrate. It was potently inhibited by placental RNase inhibitor, the selective pancreatic-type RNase inhibitor Inhibit-Ace, and a polyclonal antibody against human pancreatic RNase. The enzyme isolated from medium conditioned by immortalized umbilical vein endothelial cells (EA.hy926) possesses an amino-terminal sequence identical to that of pancreatic RNase, and shows molecular heterogeneity (molecular weights 18,000–26,000) due to different degrees of *N*-glycosylation. Endothelial cells from arteries, veins, and capillaries secreted up to 100 ng of this RNase daily per million cells, whereas levels were low or undetectable in media conditioned by other cell types examined. The corresponding messenger RNA was detected by RT-PCR in most cell types tested so far, and level of its expression was in keeping with the amounts of protein. The selective strong release of pancreatic-type RNase by endothelial cells suggests that it is endowed with non-digestive functions and involved in vascular homeostasis. J. Cell. Biochem. 86: 540–552, 2002.

Key words: endothelial cell; pancreatic ribonuclease; serum-free cell culture

Pancreatic RNase is the archetype of the RNase superfamily, of which six members have been characterized in humans. Protein sequence comparisons and enzyme activity-based analyses have led to the classification of these enzymes in four separate families, pancreatic-type RNase being designated RNase 1 in the new nomenclature [Beintema, 1998].

Although pancreatic RNase has been extensively studied at the structural and enzymatic

Abbreviations used: EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; RNase, ribonuclease; BS-RNase, bovine seminal RNase; HP-RNase, human pancreatic ribonuclease; HS-RNase C, human serum ribonuclease; RI, ribonuclease inhibitor; RNase A, bovine ribonuclease A; HUAEC, umbilical arterial endothelial cells; HuDEC, dermal microvascular endothelial cells; HuLEC, lung microvascular endothelial cells; HuMMEC, mammary microvascular endothelial cells; HPEC, placental microvascular endothelial cells; SVEC, saphenous vein endothelial cells; HUVSMC, umbilical vein smooth muscle cells.

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*Correspondence to: Michel Moenner, INSERM EPI-0113, Université Bordeaux-I, Avenue des Facultés, 33405 Talence-Cedex, France.
E-mail: m.moenner@croissance.u-bordeaux.fr

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levels, its function is not fully understood. Like related RNases, it is secreted and may therefore be considered as an ectoenzyme with regard to the intracellular location of RNA substrates. The enzyme is expressed, along with digestive proteases, by the exocrine pancreas, and a catabolic role has been postulated. However, RNase activity is far higher in the pancreas of ruminants and ruminant-like species than in that of other vertebrates, which suggests a digestive function only in certain herbivores [Beintema, 1998].

Phylogenetic studies indicate that this enzyme is one of the most rapidly evolving proteins, and it is thought that selective amino acid substitutions must have occurred for it to perform non-digestive functions [Benner and Allemann, 1989; Sorrentino, 1998]. This evolution is also reflected by gene duplication, with up to three distinct genes in ruminants, each encoded protein being predominantly located in the pancreas, the seminal plasma, or the brain [Breukelman et al., 1998].

Humans have only one such gene [Breukelman et al., 1993], and the pancreatic function of the protein is considered to be residual [Barnard, 1969; Beintema and Kleineidam, 1998]. The human enzyme exhibits significant activity against double-stranded RNA, a property shared by several pancreatic-type RNases in mammals [Sorrentino, 1998], raising a potential role in innate immune responses. This possibility was recently supported by reports of an anti-HIV replication effect [Lee-Huang et al., 1999]. Anti-viral activities are also displayed by at least three other enzymes of this superfamily, namely EDN, ECP, and onconase [Saxena et al., 1996; Rosenberg and Domachowske, 1999].

Expression studies can provide information on protein functions, and such studies of pancreatic-type RNase have been performed in humans. Both the protein and its mRNA were detected in most organ extracts. In keeping with its secretory nature, the enzyme was also present in body fluids [Weickmann and Glitz, 1982; Morita et al., 1986; Futami et al., 1997]. However, little information is available on the topologic representation of pancreatic-type RNase expression at the cellular level in tissues. Thus, the precise origin of this enzyme in most tissues is still unknown.

Isolated cells in culture are a powerful tool for examining these questions. Here, we looked at RNase expression at the protein and mRNA level in a variety of human normal and tumor cells under different culture conditions. Surprisingly, considerable variations in RNase expression were observed among the different cell types, with the strongest secretion by endothelial cells of distinct vascular and tissular origins. Strong expression of pancreatic-type RNase is therefore an inherent property of several vascular beds, suggesting that a significant part of the protein present in serum and various organs could originate from the endothelium. Our data shed new light on pancreatic-type RNase functions, which do not appear to be limited to catabolic function in the exocrine pancreas.

MATERIALS AND METHODS

Materials

Activated Sepharose 4-B, Protein A-Sepharose, SP-Sepharose, and Mono-S HR 5/5 column were from Pharmacia (Uppsala, Sweden). Vydac C4 (214 TP 54) reversed-phase column was from The Separations Group (Hesperia, CA). Recombinant human ribonuclease inhibitor (RI) was from Promega (Madison, WI). Inhibit-Ace was from 5prime->3prime (Boulder, CO). Synthetic homoribopolynucleotides poly(C), poly(U), and poly(A), chicken egg ovalbumin, bovine RNase B, human serum RNase (HS-RNase C), rabbit control serum, IgG, human lysozyme, fatty acid-free bovine serum albumin (f-BSA), and oligo$(dT)_{15}$ were from Sigma Chemical Co. (St. Louis, MO). Ovalbumin was passed through a 1-ml column of SP-Sepharose Fast Flow in 20 mM MES (2-[*N*-morpholino]-ethanesulfonic acid), 50 mM NaCl, pH 6.0 before use. Bovine RNase A and *N*-glycosidase F were from Boehringer (Mannheim, Germany). Wheat germ ribosomal RNA, native HP-RNase, and rabbit immune serum against HP-RNase were kindly provided by D.G. Glitz. Recombinant HP-RNase (rHP-RNase) was from Bachem (Bubendorf, Switzerland). RT-PCR primers for HP-RNase (forward primer 5′-GAA TCC CGG GCC AAG AAA TTC-3′; reverse primer 5′-GTG TCT CTC CTT CGG GCT GGT-3′) [Egesten et al., 1997] and glyceraldehyde phosphate dehydrogenase (GAPDH) (forward primer 5′-GAA GGT GAA GGT CGG AGT-3′; reverse primer 5′-GAA GAT GGT GAT GGG ATT TC-3′) were from Isoprim (Toulouse, France). Other compounds were obtained as previously reported [Moenner et al., 1997, 1999].

Cell Culture

Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared as described by Jaffe et al. [1973]. Cells were grown in gelatin-coated dishes in RPMI-1640/M199 medium (1:1, v/v) containing 20% FCS. Additional HUVEC cultures were prepared and maintained under distinct conditions described previously [Volin et al., 1998; Hewett et al., 2001] or obtained from ECACC (CRL 1730). Lung and mammary microvessel endothelial cells (HuLEC and HuMMEC, respectively) and pericardial mesothelial cells were grown as reported in Hewett et al. [2001]. Placental microvessel endothelial cells (HPEC) and umbilical arterial endothelial cells (HUAEC) were prepared as described by Schutz and Friedl [1996] and Ko et al. [1995], respectively. Media conditioned by umbilical vein smooth muscle cells (HUVSMC) and by skin fibroblasts (HSF-N) were kindly provided by P.F. Davies. HuDEC media were from M. Detmar. BeWo cells (ATCC, CCL-98) were grown in Ham's F12/K medium containing 15% FCS, and HT-29 cells (ATCC, HTB-38) were grown in DMEM/10% FCS. The following cells AG1523 (IMR, Camden, NJ), WS1 (CRL 1502), WI-38 (CCL 75), A549 (CCL 185), A-431 (CRL 1555), HeLa (CCL 2), U-937 (CRL 1593), H9 (ECACC 85050301), and MT4 (AIDS research and reference reagent program catalogue, 120) were propagated in FCS or in serum-free medium as described previously [Moenner et al., 1994, 1997]. Growth media were centrifuged at 12 000*g* for 5 min and buffered at pH 7.2 using a solution of 1 M MOPS (3-[*N*-morpholino] propanesulfonic acid), pH 7.2 to determine RNase contents. For RNase purification, EA.hy926 cells [Edgell et al., 1983] were grown to confluence in 576-cm^2 culture plates. Cells were washed three times in phosphate-buffered saline (PBS), incubated twice at 37°C for 15 min in DMEM, washed again in PBS and incubated for 48 h in DMEM. Twelve liters of conditioned DMEM medium were collected, centrifuged at 100*g* for 5 min and 12,000*g* for 5 min, then stored at −20°C.

RNase Assays

The radio-RNase inhibitor assay (RRIA) and catalytic assays using wheat germ RNA or yeast tRNA were performed as previously described [Moenner et al., 1997, 1999]. Substrate preference studies using poly(A), poly(C), and poly(U) were performed essentially as reported by Zimmerman and Sandeen [1965]. Briefly, homopolyribonucleotides (400 μg/ml) were added to 600 μl of RNase buffer (50 mM Tris-HCl, 130 mM NaCl, 2 mM EDTA, and 0.1 mg/ml acetylated-BSA, pH 8.0). RNase was added and incubations allowed to proceed at 37°C. At various times, 100-μl aliquots were mixed with 250 μl of ice-cold 6% perchloric acid, 20 mM Lanthanum chloride. Then, 100 μl of a 10 mg/ml f-BSA solution was added and mixtures were maintained on ice for 15 min and centrifuged for 15 min at 16,000*g* at 4°C. Substrate degradation was determined by measuring the absorbance of the supernatant at 260 nm (poly(A) and poly(U)), or at 280 nm (poly(C). Bovine RNase A and native HP-RNase concentrations were determined by absorption spectrophotometry at 278 nm ($\varepsilon = 9\ 800\ M^{-1}cm^{-1}$) and by analyzing the amino acid composition, respectively. The EC-RNase concentration was determined by RRIA using HP-RNase as standard. Inhibitory units for RI and Inhibit-Ace are those given by the suppliers. Assays were performed in duplicate except where indicated otherwise.

Purification and Characterization of RNase From EA.hy926 Cell-Conditioned Medium

The RNase concentration was monitored during purification by using RRIA, and by measuring catalytic activity and its inhibition by antibodies to HP-RNase. Culture medium conditioned by EA.hy926 cells was diluted twofold with 10 mM MES, pH 6.0 and applied to a 5 × 3-cm column of SP-Sepharose Fast Flow at a flow rate of 10 ml/min at 4°C. The column was then washed in 20 mM MES, 50 mM NaCl, pH 6.0, and RNase was eluted at a flow rate of 4 ml/min in the same buffer adjusted to 1 M NaCl and containing 100 μg/ml ovalbumin. The effluent was dialyzed against 10 mM ammonium acetate, pH 8.2, and lyophilized. The preparation was reconstituted in the same buffer, clarified by centrifugation (250,000*g*, 30 min) at 4°C, and filtered through a Millex-GV4 0.22-μm filter (Millipore) before being applied to a Mono-S HR 5/5 column at a flow rate of 1 ml/min. Proteins were eluted using a 45 mn-linear gradient (10–600 mM) of ammonium acetate. In these conditions, 74% of total RNase activity was eluted between 8 and 13 min. The corresponding fractions were pooled, lyophilized, and resuspended in 0.1% TFA. Proteins were loaded on a C4 reverse-phase column and eluted using

a linear gradient of 10–60% CH_3CN in 0.1% TFA over 33 min at a flow rate of 1 ml/min. The protein concentration in non-homogeneous protein samples was determined by using the Bio-Rad assay (Bio-Rad) with BSA as standard. The concentration of pure EC-RNase was determined by RRIA and RNase assays and is given in nanograms-equivalent of a native HP-RNase standard preparation. Protein microsequencing was performed by J. D'Alayer at the Département des Biotechnologies, Institut Pasteur, Paris. Purified 18-kDa EC-RNase was subjected to microsequencing on an Applied Biosystems 473A sequencer after elution from the reverse-phase C4 column. Sequence comparisons were performed using the BLAST algorithm [Altschul et al., 1990].

Treatment With *N*-Glycosidase F

EC-RNase purified by C4 reverse-phase chromatography was lyophilized, resuspended in water, and again lyophilized. One microgram of protein was then solubilized in water and incubated with 1 U of *N*-glycosidase F for 18 h in 200 mM sodium phosphate, 20 mM EDTA, 1% β-mercaptoethanol, 0.1% sodium dodecyl sulphate (SDS), 0.5% NP40, pH 7.4 at 37°C. The digested EC-RNase was analyzed by SDS–polacrylamide gel electrophoresis (SDS–PAGE) with silver nitrate staining.

Immunoprecipitation

Rabbit immune serum against HP-RNase was passed through a column of bovine RNase A (3 mg) coupled to Sepharose 4-B (1 ml). The flowthrough fractions contained antibodies that recognized HP-RNase but neither bovine RNase A nor RNase B. Immunoprecipitation was performed as follows. Protein A-Sepharose beads were preincubated overnight at 4°C in MOPS-buffered saline (MBS, 20 mM MOPS, 130 mM NaCl, 3 mM KCl, pH 7.4), 2% f-BSA. Beads were washed twice in washing buffer (MBS, 0.2% f-BSA), twice in 20 mM sodium acetate, pH 2.5 and twice in MBS. Fifty microliters of beads were incubated for 90 min with 40 μl of immune or non-immune serum. Beads were washed three times in washing buffer and mixed gently in 2-ml polypropylene tubes with 1.8 ml of cell supernatants overnight at 4°C. Supernatants were discarded and beads were washed twice in washing buffer and twice with 50 mM NaCl, pH 7.00 (final volume 100 μl). In a first set of experiments, 50 μl non-reducing Laemmli buffer (3×) was added and samples were boiled for 3 min. Forty-microliter aliquots of each sample were then electrophoresed for immunoblotting analysis. Alternatively, RNase-antibody complexes were dissociated by incubating beads for 4 h in 3.2×10^{-2} M HCl, 50 mM NaCl at 37°C. Media were then buffered with 10 volumes of 0.5 M Tris-HCl, 130 mM NaCl, pH 8.00, and ribonucleolytic activity was measured.

SDS–PAGE and Immunoblotting

After migration on 15% SDS–PAGE, proteins were either revealed by silver staining or transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad Laboratories, Melville, NY). Prelabeled molecular weight markers were used as migration standards. The membrane was saturated overnight in 10 mM Tris, pH 7.4, 4% low-fat milk at 4°C. Immune serum was then incubated at room temperature for 4 h at 1/50 dilution in the same buffer. The membrane was washed and incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (HRP; Diagnostics Pasteur) for 1 h at room temperature. Peroxidase activity was revealed by using the SuperSignal Substrate (Pierce, Rockford, IL) followed by autoradiography. Quantitative analyses were performed by using Molecular Analyst software from Bio-Rad.

Semi-Quantitative RT-PCR

Cells were grown to confluence in 78-cm^2 dishes in the conditions described above. Total cellular RNA was extracted by using the Trizol reagent kit (Life Technologies) and quantified with a UV spectrophotometer. RNA was reverse-transcribed with two different methods, the first using primers specific for GAPDH or HP-RNase genes and the second using oligo$(dT)_{15}$. The two methods yielded similar results as to the relative expression of HP-RNase in different cell types; however, the second method was preferred, as it provided a more accurate semi-quantitative estimate: 10 μg total RNA was denatured at 65°C for 5 min, then reverse transcribed in a final volume of 40 μl containing 1× SuperScript first-strand buffer, 10 mM DTT, 25 μg/ml oligo$(dT)_{15}$, 0.5 mM each deoxyribonucleoside triphosphate (dNTPs), and 400 U of SuperScript RNase H reverse transcriptase (Life Technologies). The reaction was run for 50 min at 42°C and then stopped by heating at 70°C for 15 min. After reverse

transcription, 3.3 μl (for GAPDH) or 10 μl (for HRP) of template cDNA was subjected to PCR amplification using 100 pmol of each primer and 2 U of *Taq* DNA polymerase (Life Technologies) in the PCR mixture containing 1 × *Taq* buffer, 1.5 mM $MgCl_2$, and 0.2 mM each deoxyribonucleoside triphosphate (dNTPs) in a total volume of 50 μl. PCR amplification of human GAPDH began with a denaturing step at 94°C for 10 min, then 21 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a final extension step of 10 min at 72°C. The protocol for HP-RNase was similar, except that it involved 28 cycles and an annealing temperature of 63°C. Quantification was performed using NIH-image software version 1.62.

RESULTS

Expression of Pancreatic-Type RNase by Cells in Serum-Free Medium

RNase expression was measured in human cells grown or maintained in serum-free conditions. Cell-conditioned media were checked for their RNase content by radio-competition assay measuring ^{125}I-angiogenin binding to RI [Moenner et al., 1999]. The assay was performed in the presence or absence of an excess of Inhibit-Ace, a RNase inhibitor that interacts specifically with RNases of the pancreatic type. In such conditions, pancreatic-type RNases can easily be distinguished from non-pancreatic-type RNases (Fig. 1). The RNase content was also evaluated on the basis of degradation of wheat germ RNA and by measuring its sensitivity to anti-HP-RNase antibodies.

As shown in Table I, HUVEC and EA.hy926 cells (a hybrid obtained by fusing HUVEC with A549/8 carcinoma cells [Edgell et al., 1983]) secreted large amounts of RNase activity, most of which was sensitive to Inhibit-Ace and to polyclonal antibodies against HP-RNase. Like their parental HUVEC, EA.hy926 cells therefore expressed a pancreatic-type RNase, while the fused tumor cell partner (A549/8) did not. In a distinct assay, HUVEC and EA.hy926 (but not A549/8) cell-conditioned media were found to contain ribonucleolytic activity that degraded tRNA and was inhibited by both Inhibit-Ace and RI (Fig. 2). This activity was also detected in media conditioned by other human cells, although at a much lower level. The enzyme

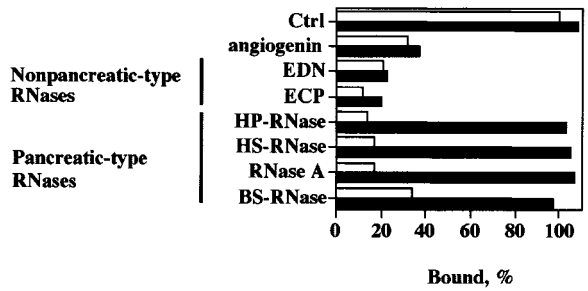

Fig. 1. Inhibit-Ace prevents binding of pancreatic-type RNases to RI. The inhibitory effect of Inhibit-Ace on the binding of RNases to RI was measured by radio-RI assay (RRIA) [Moenner et al., 1999]. ^{125}I-angiogenin (7,000–10,000 cpm) was pre-incubated for 10 min with or without 6 U of Inhibit-Ace and in the presence or absence of angiogenin (2 ng), RNase A (2 ng), EDN (20 ng), ECP (20 ng), native HP-RNase (4 ng), BS-RNase (40 ng), or HS-RNase C (3.5 U). RI (0.015 U) was then added for 10 min (total volume 480 μl) and the interaction of the free fraction of ^{125}I-angiogenin with RI was quantified after precipitation in 50% ammonium sulfate. The binding of ^{125}I-angiogenin to RI is reported in abscissa in the presence (closed bars) or absence (open bars) of Inhibit-Ace. All values were obtained from duplicate experiments that differed by less than 5%.

TABLE I. Presence of RNases in Serum-Free Media Conditioned by Human Cells

		RRIA		RNase activity	
Cells	Medium complement	RNase (ng-eq./10^6 cells/day)	% Inhibition (Inhibit-Ace)	RNase (ng-eq./10^6 cells/day)	% Inhibition (Ab. to HP-RNase)
HUVEC	HDL + FGF	158 ± 31	≥96	211 ± 37	≥98
EA.hy926	HDL	142 ± 41	≥80	233 ± 43	≥95
A549/8	HDL	5.3 ± 1.1	NS	2.3 ± 0.3	NS
A-431	None	8.5 ± 1.8	<5	9.9 ± 1.1	<11
HT29	None	38 ± 6	<36	28.0 ± 1.6	<7

Cells were grown in the presence of serum until confluent. They were washed three times with PBS and incubated for 36 or 48 h in serum-free culture medium. The medium consisted of DMEM, 1 mg/ml f-BSA to which 150 μg/ml HDL and 2 ng/ml FGF-2 were added as indicated. RNase amounts in conditioned media were measured by RRIA and RNase assays using wheat-germ RNA as substrate. The control medium (DMEM/HDL/FGF) contained no detectable RNase as determined by RRIA (< 0.4 ng/ml RNase), catalytic assay (< 20 pg/ml RNase), and RIA (< 20 pg/ml angiogenin). Values were obtained from triplicate assays performed on at least two independent cultures. Polyclonal antibodies against HP-RNase were purified on a protein A-Sepharose column before use. The results are expressed in nanogram-equivalents (ng-eq.) of a standard HP-RNase preparation. NS, not significant.

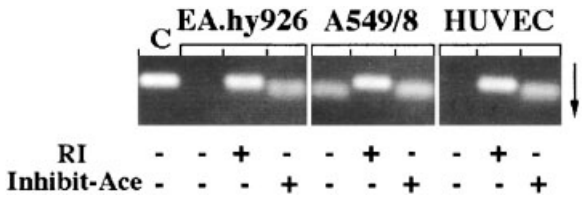

Fig. 2. Catalytic activity toward tRNA of the pancreatic-type RNases expressed by HUVEC and EA.hy926 cells. RNase activity was measured in media conditioned by HUVEC, A549/8, and EA.hy926 cells. Cells were cultured for 36 h (see Table I) and cell-conditioned media collected. One microgram of tRNA was incubated for 30 min at 37°C with 16 µl of cell-conditioned medium in the presence or absence of either RI (2 U) or Inhibit-Ace (1 U). **Lane C** corresponds to the experiment with control medium. Arrow indicates the direction of migration of tRNA. Amounts of RNase-related material present in 16 µl of medium were determined by RRIA and corresponded to 0.63 ± 0.12, 1.14 ± 0.33, and 0.04 ± 0.008 ng angiogenin-eq. with HUVEC, EA.hy926, and A549/8 cells, respectively.

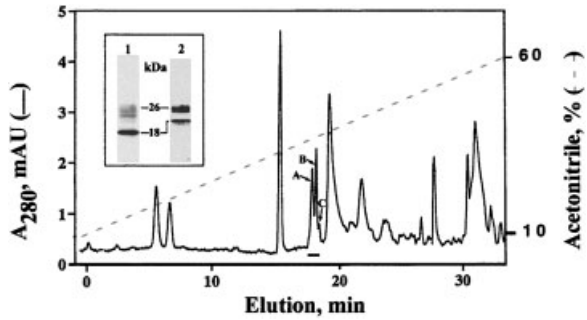

Fig. 3. Purification of EC-RNase. Reverse-phase HPLC purification of EC-RNase protein recovered from Mono S column. A Vydac C4 column was equilibrated in 10% CH_3CN, 0.1% TFA, and eluted with a 40-min linear gradient of 10–65% CH_3CN at a flow rate of 1 ml/min. Fractions underlined with a solid bar contained 80% of the total RNase activity using wheat germ ribosomal RNA as substrate. The inset corresponds to electrophoretic profiles of the purified protein (peaks **A** and **B**) after migration on SDS–PAGE and detection by silver nitrate staining (**lane 1**) or immunoblotting with anti-HP-RNase antibodies (**lane 2**). Molecular weights are indicated.

released by HUVEC and EA.hy926 cells is subsequently referred to as endothelial cell-derived RNase (EC-RNase).

Purification and Characterization of EC-RNase From EA.hy926 Cell-Conditioned Medium

EC-RNase was purified from EA.hy926 cell supernatants by using SP-Sepharose fast flow, Mono-S chromatography and C4 reverse-phase HPLC. The last step allowed us to resolve two absorbance peaks (A–B) that eluted at 29–30% acetonitrile and contained proteins with molecular masses in the 18- to 26-kDa range (Fig. 3; inset, lane 1). A similar pattern of migration was observed with and without reduction (data not shown). These proteins were cross-reactive with antibodies to HP-RNase (Fig. 3; inset, lane 2). In addition, they exhibited potent ribonucleolytic activity which was totally inhibited by both RI and Inhibit-Ace. Peak C corresponded to a minor ~28-kDa protein that was not present in all preparations and whose amino-terminal sequence was unrelated to that of any characterized RNases (data not shown). Further runs through C4 reverse-phase columns of peaks A and B pooled from five different separations resolved the enzyme in at least three peaks (Fig. 4A). Electrophoretic analysis indicated that the component with the highest molecular weight eluted in the first peak, while the last peak consisted of an 18-kDa protein. Amino-acid sequence analysis showed that the ten NH_2-terminal residues of the 18-kDa protein were identical to those of HP-RNase (Fig. 4B).

EC-RNase Molecular Heterogeneity Due to Variable *N*-Glycosylation

The molecular heterogeneity observed with EC-RNase has already been described with pancreatic-type RNases purified from various tissues and shown to result from different degrees of glycosylation [Beintema et al., 1988; Ribo et al., 1994]. This suggested that EC-RNase could also be differentially glycosylated. In order to confirm this, purified EC-RNase was incubated with *N*-glycosidase and analyzed by SDS–PAGE. After digestion, the purified enzyme migrated essentially as an 18-kDa band, indicating that *N*-glycosylation indeed generates the observed molecular heterogeneity (Fig. 4C).

Substrate Preferences of EC-RNase and Inhibition by RNase Inhibitors

The ribonucleolytic activity of EC-RNase was tested in comparison with that of HP-RNase and bovine RNase A. In our assay conditions, the three proteins cleaved poly(C) at approximately 35 times the rate of poly(U), while no activity was observed towards poly(A) (Table II). EC-RNase and HP-RNase also had similar activity against wheat germ RNA substrate, but this activity clearly differed from that of RNase A: the relative rate of degradation of poly(C) to RNA by the human proteins were approximately 17-fold lower than that obtained with

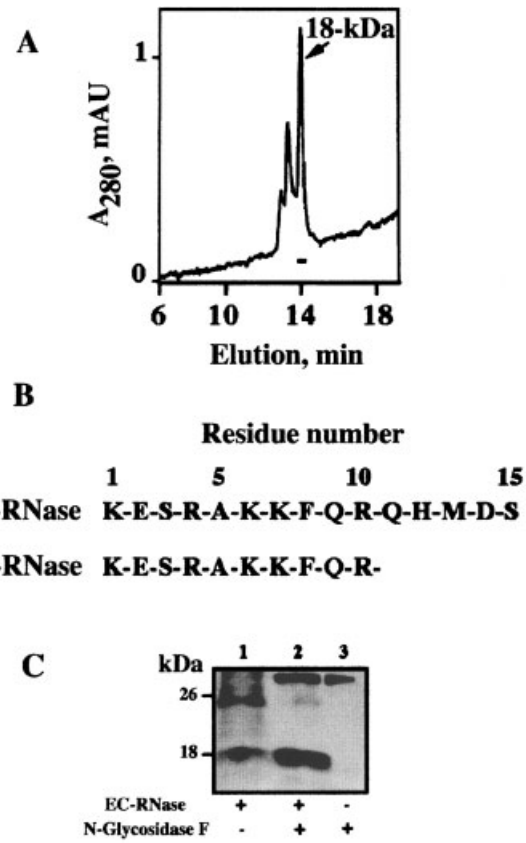

Fig. 4. Molecular characteristics of EC-RNase. (**A**) Analytical run of EC-RNase on RP-HPLC. Purified EC-RNase was injected on the reverse-phase C4 column equilibrated in 25% CH_3CN, 0.1% TFA and eluted with a 20-min linear gradient of 25–50% CH_3CN at a flow rate of 1 ml/min. The fraction corresponding to the 18-kDa protein (solid bar) is indicated. (**B**) The amino-terminal sequence obtained for the 18-kDa EC-RNase and the reported sequence of HP-RNase [Beintema et al., 1984] are shown for comparison. (**C**) Peaks A-B in Fig. 3 were pooled and EC-RNase was digested for 18 h with *N*-glycosidase F and analyzed by SDS–PAGE and silver staining. **Lane 1**, EC-RNase; **Lane 2**, EC-RNase incubated with *N*-glycosidase F; **Lane 3**, *N*-glycosidase F alone.

TABLE II. Substrate Specificities of Human EC-RNase, HP-RNase, and Bovine Pancreatic RNase A

	O.D.260/ng enzyme/ml/min (relative rate of degradation, %)		
Substrate	RNase A	HP-RNase	EC-RNase
Poly(C)	1.350 (100)	1.147 (100)	1.187 (100)
Poly(U)	0.039 (2.9)	0.027 (2.4)	0.030 (2.5)
Poly(A)	ND	ND	ND
WGRNA	0.913 (68)	0.062 (5.4)	0.055 (4.6)

The RNase activity was determined by the formation of acid-soluble nucleotides. Values obtained with Poly(C) substrate were defined as 100%. ND, not detectable. WGRNA, wheat germ RNA.

bovine RNase. The 18-kDa EC-RNase and rHP-RNase also have the same activity profile (data not shown), indicating that the glycosylation pattern does not significantly interfere with enzyme activity.

The catalytic activities of both HP-RNase and EC-RNase were abrogated by RI and Inhibit-Ace (Table III). Antibodies directed against HP-RNase also inhibited both enzymes, but were relatively inactive on bovine RNase A. Taken together, the RP-HPLC purification profiles, sequence identities, catalytic potencies and sensitivities to protein inhibitors and antibodies suggested that the catalytic sites and overall structure of enzymes isolated from human endothelial cells and pancreas possess high structural homologies. No modulation of the enzyme activity was detected in relation to its tissue origin.

Endothelial Cell-Specific Expression of Pancreatic-Type RNase

The expression of pancreatic-type RNase was further examined in a variety of cells grown in the presence of serum as a number of human cell lines do not withstand serum-free culture conditions. Cell-conditioned media were subjected to immunoprecipitation with polyclonal antibodies against HP-RNase, and the enzyme was then detected by immunoblotting. In agreement with our results obtained with serum-free cul ture, HUVEC and EA.hy926 cells secreted immunoreactive proteins while A549/8 cells did not (Fig. 5A). The observed protein heterogeneity (three major bands;

TABLE III. Inhibition of EC-RNase With RI, Inhibit Ace, and Anti-HP-RNase Antibodies

	% Inhibition		
RNase	RI	Inhibit-Ace	HP-RNase antibodies
RNase A	98	95	13
EC-RNase	97	86	94
HP-RNase	99	90	95

The catalytic activities of the EC-RNase, HP-RNase, and bovine RNase A were measured using wheat germ RNA. The quantities of the enzymes used were chosen to give an $O.D._{260}$ of 0.70–0.82 after 45-min incubation at 37°C. Inhibition was obtained by adding RI (6 U/ml), Inhibit-Ace (6 U/ml), or affinity-purified polyclonal antibodies against HP-RNase (50 ng). RNase activity is reported as the percentage inhibition of the maximal value (100%) obtained in the absence of inhibitor. Each duplicate value differed by less than 5%.

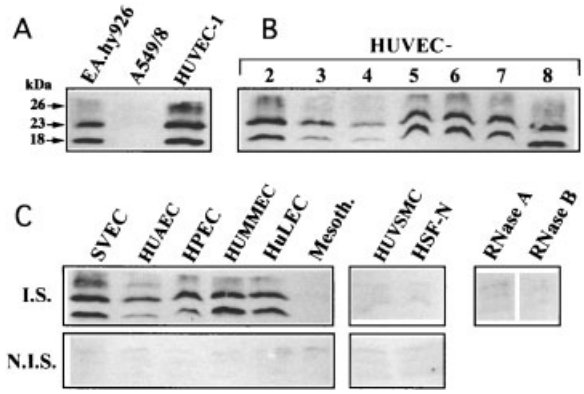

Fig. 5. Expression of pancreatic-type RNase immunoreactivity by cells grown in the presence of serum. Cells were grown in the presence of serum and supernatants collected after 2 days of incubation, except for HUVEC-1 (3 days) and HPEC (1 day). Supernatants were subjected to immunoprecipitation followed by immunoblotting with polyclonal antibodies directed against HP-RNase. (**A**) Expression of RNase by the hybridoma EA.hy926 cells was compared to that of the two parent cells (HUVEC and A549/8). (A–**B**) Expression of RNase by HUVEC obtained from different subjects and subcultured in different laboratories. HUVEC-1, -3, and -4: primary culture with supernatants from day 2–5, 1–3, and 0–2, respectively. HUVEC-2: confluent cells from the ATCC, passage 18; HUVEC-5 to -8: confluent cultures at passage 2, 6, 6 and 7, respectively. (**C**) **Upper panel**: comparative expression of pancreatic-type RNases by endothelial and non-endothelial cells from different tissues. HuDEC do not express the immunoreactivity (data not shown). Controls using immune serum with 100 ng of either bovine RNase A or RNase B are indicated on the right. **Lower panel**: control experiments performed with non-immune serum (NIS). For comparison, all samples are from the same immunoprecipitation experiment run in parallel on SDS/PAGE. Transfer and immunoblotting were performed on the same nitrocellulose membrane.

Mr: 18–26 kDa) was consistent with that of purified EC-RNase.

The degree of RNase expression by HUVEC was unrelated to the number of subcultures. Cells in primary culture (HUVEC-1, -3 and -4), grown for 3–6 passages (HUVEC-5 to -8) or for more than 18 passages (HUVEC-2; CRL 1730) expressed similar immunoreactivity (Fig. 5B). RNase was secreted at all growth stages, including the lag phase, the exponential growth phase, and at confluence. An effect of HUVEC heterogeneity in different cultures [Watson et al., 1995] was ruled out, as cells isolated from several individuals and cultured in different laboratories (HUVEC-1 to -8) gave similar results.

Endothelial cells are morphologically and functionally heterogenous according to their vessel and tissue origins [Bassenge, 1996]. Production of pancreatic-type RNase was therefore examined in cells from different blood vessels and organs. As shown in Figure 5C, endothelial cells from umbilical arteries, saphenous veins, and lung, mammary, and placenta microcapillaries also released this immunoreactivity. Expression of pancreatic-type RNase is therefore a property shared by numerous endothelial cells. In comparison, non-endothelial cells, including HUVSMC, pericardial mesothelial cells, A549/8 tumor cells and HSF-N fibroblasts, did not secrete detectable amounts of this protein. Densitometric analyses showed that the most prominent band was frequently that migrating at ~23 kDa, although slight variations were observed among different HUVEC and endothelial cell cultures.

The catalytic characteristics of RNases detected in different endothelial cell supernatants were also compared. As human pancreatic-type RNase is resistent to acid treatment [Sierakowska and Shugar, 1977], a low pH was applied to immunopurified RNase samples in order to inactivate inhibiting antibodies and to release the active enzyme. RNase assays were then performed using poly(A), poly(C), and poly(U) substrates and in the presence or absence of RNase inhibitors. The results in Table IV confirm the selective presence of the pancreatic-type RNase in media conditioned by endothelial cells. These results, together with those in Figure 5, show that all the EC-derived pancreatic-type RNases are identical on the basis of the following criteria: (i) molecular size and heterogeneity; (ii) cross-reactivity to antibodies against HP-RNase; (iii) catalytic preference, cleaving poly(C) better than poly(U) and barely cleaving poly(A); (iv) sensitivity to both RI and Inhibit-Ace; and (v) resistance to acid treatment. The amounts of RNase were lower than expected from experiments in serum-free culture (see Table I). Real values may have been higher, as the acid treatment yielded only 40% recovery of the initial activity (control assay not shown).

Seven types of endothelial cells expressed significant amounts of the protein (Table V). Most non-endothelial cells did not secrete detectable amounts of RNase, including cells from the immune system, vascular smooth muscle cells, fibroblasts, epithelial cells such as pericardial mesothelial cells, and several carcinoma cells. HT-29, A431, and WI-38 and WS1 cells expressed the enzyme at a low level.

TABLE IV. Expression of Pancreatic-Type RNase by Different Cell Types Grown in the Presence of Serum

Cell	RNase expression ng/10^6cell/day	Activity poly(C)/poly(U)	Inhibition by RI (%)	Inhibition by Inhibit-Ace, (%)
EA.hy926	56	35	99	80
HUVEC	88	37	99	86
SVEC	14	19	99	84
HUAEC	13	46	93	85
HPEC	23	42	100	78
HuLEC	52	38	95	89
HuMMEC	47	41	96	92
HuDEC	ND	—	—	—
Mesothelial	ND	—	—	—
BeWo	ND	—	—	—
HUVSMC	ND	—	—	—
HuVS-fib	ND	—	—	—
HSF-N	ND	—	—	—
AG1523	ND	—	—	—
WI-38	0.1	—	—	—
U-937	ND	—	—	—
MT4	ND	—	—	—

Catalytic activity, substrate preference, and sensitivity to RNase inhibitors.
The RNase was purified from supernatants by immunoprecipitation using anti-HP-RNase antibodies. RNase-antibodies complexes were then dissociated by acid treatment, and degradation of polynucleotides was determined by measuring acid-soluble products as described in Materials and Methods. Enzymatic activity was measured at 37°C in the presence or absence of RI (2 U) or Inhibit-Ace (1 U). RNase was quantified using a poly(C) substrate and results are reported in nanogram-equivalents of a standard preparation of HP-RNase. Experiments using either unincubated control media with immune serum or cell-conditioned media with non-immune serum gave negative results. No activity was detected using a poly(A) substrate for either cell type. At least two immunoprecipitation assays were performed for each conditioned medium, and for comparison the results presented are from the same experiment. Values were obtained from duplicate assays and differed by less than 20%. Percent inhibition differed by less than 5%. ND, not detectable.

RT-PCR Experiments

Semiquantitative RT-PCR experiments were performed to assess the expression of the RNase gene in five cell types expressing the enzyme activity at levels ranging from undetectable to high. Given the molecular identity observed between EC-RNase and HP-RNase, primers for the pancreatic enzyme were used. PCR experiments were performed between cycles 20 and 30 for RNase and between cycles 17 and 25 for GAPDH. Linearity of PCR amplification was observed up to 23 cycles for GAPDH (all cell types) and up to 28 cycles for HP-RNase (HUVEC and EA.hy926 cells). Cycles 21 and 28 were thus chosen for direct comparison of the relative expression of these genes. As shown in Figure 6, both HUVEC and EA.hy926 cells strongly expressed the RNase mRNA, whereas A549/8 cells did not express it. A faint signal was observed with A431 and HT29 cells, becoming clear at cycles 30–35 (data not shown). These results are in agreement with those obtained at the protein level.

DISCUSSION

Proteins of the pancreatic ribonuclease superfamily are secreted enzymes with pleiotropic biological activities. Among members of this superfamily, EDN and/or ECP have been shown to possess anti-viral, anti-bacterial, and

TABLE V. Expression of Pancreatic-Type RNase by Endothelial Cells

Cell type	Number of positive cells	Name
Epithelial	1/7	Pericardial mesothelial; BeWo; [a]HT-29; A-431; A549; A549/8; HeLa
Endothelial	7/8	HUVEC; EA.hy926; HUAEC; SVEC; HPEC; HuLEC; HuMMEC; HuDEC
Fibroblast	0/5	HSF-N; HuSVF; WS1; WI-38; AG1523
Smooth muscle cells	0/2	HUVSMC; AALTR 16.2
Immune cells	0/3	MT4; H9; U-937

Results obtained with different human cell types. The pancreatic-type RNase was measured in serum-free medium as described in Table I, or in serum-containing media as reported in Figure 5 and Table IV. Cell types were considered positive when they expressed >5 ng/10^6 cells per day. The names of tumor and immortalized cells are underlined.
[a]HT-29 cells expressed <10 ng/10^6 cells of pancreatic-type-RNase per day.

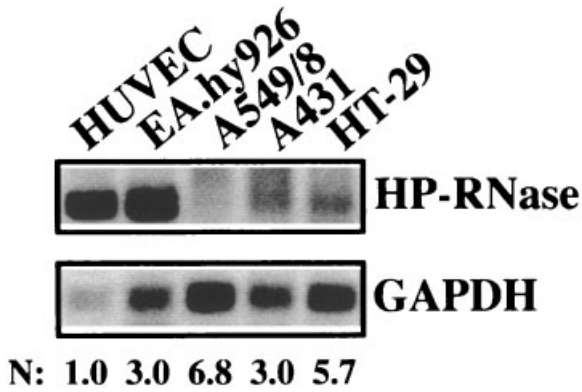

Fig. 6. RT-PCR. Semi-quantitative RT-PCR with 28-cycle amplification of HP-RNase mRNA and 21-cycle amplification of GAPDH mRNA were performed as described in Materials and Methods. PCR products were resolved on a 1.5% agarose gel containing ethidium bromide. GAPDH was used as the internal control. Signal intensities of GAPDH bands were quantified and normalized (N), the 1.0 value being used as the reference. Two independent cultures were analyzed for each cell type, with similar results.

anti-parasitic activities, and induce the Gordon phenomenon in rabbits [McLaren et al., 1984; Gleich et al., 1986; Rosenberg, 1995; Rosenberg and Domachowske, 1999]. Angiogenin triggers neovascularization in vivo and exhibits various activities towards cells in culture [Strydom, 1998]. Pancreatic-type RNases contribute to the digestive process in certain herbivores [Barnard, 1969] and may also have aspermatogenic, immunosuppressive, anti-viral, and anti-tumoral actions [D'Alessio et al., 1991; Lee-Huang et al., 1999].

Tissue expression of these enzymes appears to be more or less restricted. Pancreatic-type RNase is ubiquitous in human tissues [Weickmann and Glitz, 1982; Morita et al., 1986; Futami et al., 1997], but the local cellular contribution to its synthesis is unknown. In this work, endothelial cells were found to secrete large amounts of an RNase that was indistinguishable from HP-RNase by means of biochemical, immunological, and enzymatic methods. As there is no evidence for more than one gene coding for RNase 1 in humans, the endothelial cell- and pancreas-derived proteins are very likely identical. In addition, these enzymes were not subjected to selective protein modification that modulated their activities depending on the tissue origin.

Endothelial cells from large and small blood vessels secreted the pancreatic-type enzyme at a high rate, which suggests that it is expressed by several types of endothelium and is not organ-specific. According to the estimated surface area and weight of the endothelial lining in humans ($\sim$1000 m^2; 1.5 Kg) [Jaffe, 1987; Bassenge, 1996], milligram amounts of RNase should be expressed per day in this tissue assuming that levels of RNase secretion in cultured endothelial cells are representative of those in vivo. Pancreatic-type RNase expression was also much more cell-restricted than expected, as most of the other cells tested to date exhibit little or no detectable activity. Among these, mesothelial cells share several characteristics with endothelial cells [Hewett et al., 2001], while Bewo choriocarcinoma cells are derived from trophoblasts, which are known to acquire an endothelial cell phenotype during placental development [Redman, 1997]. Despite their relationship with endothelial cells, these two latter cell types did not express pancreatic-type RNase. Cells from the immune system (MT4, H9, and U937) did not release detectable amounts of the enzyme either, in agreement with mRNA analyses reported by others [Schein et al., 1992; Futami et al., 1997].

Pancreatic-type RNase and angiogenin are major RNases in the blood circulation, with median serum concentrations of $\sim$400 ng/ml and $\sim$250 ng/ml, respectively [Weickmann et al., 1984; Bläser et al., 1993]. However, the sites of synthesis of these two related enzymes clearly differs: angiogenin is mostly expressed in liver [Weiner et al., 1987], whereas this organ does not contribute significantly to the release of pancreatic-type RNase [Zhao et al., 1998]. In addition, expression of angiogenin by human endothelial cells is at least 50-fold lower than that of pancreatic-type RNase ([Moenner et al., 1994] and this work).

Although it is not yet known whether our results may be fully transposed to the in vivo situation, the high rate expression of pancreatic-type RNase seems to represent an endogenous property of endothelial cells if we consider the number of cell types tested. The observed secretion by early endothelial cell primary cultures also supports this hypothesis. These results would therefore provide an explanation for earlier reports questioning the pancreatic origin of pancreatic-type RNases in serum and urine. Pancreatectomy in both rats and humans does not induce significant variations in the amount of serum RNase [Rabinovich and Dohi, 1956; Peterson, 1979],

and RNase activity in the serum of different animal species is unrelated to its expression in the pancreas [Lechner and Magalhaes, 1973]. The detection of high levels of pancreatic-type RNase activity in human aorta and femoral artery, relative to other tissues, including pancreas and kidney [Oribe et al., 1986], is also in agreement with this hypothesis.

From the post-translational point of view, EC-RNase occurs as a heterogenously glycosylated protein that might be identical to serum- and to urinary-RNases as a direct consequence of its release by endothelium into the blood stream and subsequent renal clearance. Besides, EC-RNase and HP-RNase should have different carbohydrate compositions, based on consistent antigenic differences between pancreatic and serum pancreatic-type RNases [Neuwelt et al., 1977; Yamashita et al., 1986] and between urinary and pancreatic RNases [Beintema et al., 1988], respectively.

Although these results show strong selective expression of the pancreatic-type RNase by endothelial cells, this protein cannot be considered as a marker of these cells. Indeed, epithelial cells such as acinar cells from the pancreas and parotid express the protein in other species [Sierakowska and Shugar, 1977]. Pancreatic tumor cells [Fernandez-Salas et al., 2000] and other cells (this work) also share this property in vitro, even if the amounts of RNases detected were much lower than those produced by endothelial cells.

The biological significance of the high rate of EC-RNase production is not known, although it is unlikely to be related to a digestive function. Like other RNases of the same family, human pancreatic-type RNase has potent anti-viral activity [Lee-Huang et al., 1999]. A protective role of the protein against virus spread might therefore be expected. The expression rate of EC-RNase, and its serum concentration, are consistent with this possibility. The presence of RNase in urine, saliva, and seminal fluid is also in agreement with an anti-microbial function. The strong specific expression by endothelial cells might target intravascular pathogens. As ECP possess anti-bacterial activities [Rosenberg, 1995], we tested pancreatic-type RNase for such activity. However, neither liquid nor solid medium assays showed any bactericidal or bacteriostatic activity on several commensal bacteria, even in conjunction with lysozyme (data not shown).

Finally, a modulation of pancreatic-type RNase secretion would be expected to occur in pathological conditions associated with vascular injury. Serum from patients with rheumatoid arthritis and vasculitis contained markedly increased amounts of pancreatic-type RNase activity. Interestingly, these sera increased pancreatic-type RNase expression by HUVEC in culture, suggesting the presence of an inducible factor associated with this pathological state [Oribe et al., 1986]. These findings raise important clinical perspectives for this protein. Endothelial cell dysfunction resulting from exposure to various stimuli (atherogenic factors, microorganisms, metabolic defects, and immune-mediated injury) is a possible source of RNase plasma variations.

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